DEFENCE R&D DÉFENSE

Chinese Hamster Ovary Cell/Hypoxanthine-Guanine Phosphoribosyl Transferase Gene Mutation Assay for Reactive Skin Decontamination Lotion *(Final Report)*

Ram D. Mehta
Prairie Biological Research Ltd.

Scientific Authority:
Thomas Sawyer
Defence Research Establishment Suffield

"The use of this information is permitted subject to recognition of proprietary and patent rights."

DEFENCE RESEARCH ESTABLISHMENT SUFFIELD

Contract Report
DRES CR 1999-009

February 1999

Défense nationale National Defence

Canada

Prairie Biological Research Ltd.

PBR PRAIRIE BIOLOGICAL RESEARCH LTD.

FINAL REPORT

(DRES SOLICITATION NO. W7702-6-R599/A)

STUDY TITLE:

CHINESE HAMSTER OVARY CELL/ HYPOXANTHINE–GUANINE PHOSPHORIBOSYL TRANSFERASE GENE MUTATION ASSAY FOR REACTIVE SKIN DECONTAMINATION LOTION

PREPARED BY: **Ram D. Mehta, Ph.D. P.Biol.**

PREPARED FOR: DEFENCE RESEARCH ESTABLISHMENT LTD.
P.O. Box 4000
Medicine Hat, Alberta
Canada T1A 8K6

Attn: Dr. Thomas Sawyer

COMPLETION DATE: OCTOBER 29, 1998

STUDY NUMBER: 970805/4

DATE: December 8, 1998

Ram D. Mehta, Ph.D. P.Biol.
Manager Research and Services

4290 - 91A Street, Block C, Edmonton, Alberta, Canada T6E 5V2 • Telephone (403) 450-3957 • Fax (403) 450-3960
E-mail: pbr@oanet.com Web Site: www.ispex.ca/pbr/

CHINESE HAMSTER OVARY CELL/HYPOXANTHINE–GUANINE PHOSPHORIBOSYL TRANSFERASE GENE MUTATION ASSAY FOR REACTIVE SKIN DECONTAMINATION LOTION

Authorized Representative:	**Dr. Thomas Sawyer**
Contract Laboratory:	**Prairie Biological Research Ltd.** **4290-91A Street, Block C** **Edmonton, Alberta** **Canada T6E 5V2**
Test Substance:	**Reactive Skin Decontamination Lotion**
Test Article Lot No.:	**Lot 5D11449**
PBR Study No.:	**970805/4**
Test Substance Description:	**yellow colored lotion**
Storage Condition:	**under refrigeration (3-6°C)**
Test Article Receipt:	**August 05, 1997**
Study Initiation:	**September 26, 1998**
Study Completion:	**October 29, 1998**
Study Director:	**Ram D. Mehta, Ph.D. P.Biol.**
Analysts:	**Seema Gupta, Ph.D.** **Ebenezer Sowa**

STATEMENTS OF COMPLIANCE

Prairie Biological Research Ltd.'s Study N0. 970805/4 was conducted in compliance with the OECD Principles of Good Laboratory Practice (GLP), in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test or control article were not determined by the testing facility.

The stability of the test or control article under the test conditions was not determined by the testing facility.

Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not determined by the testing facility.

Ram D. Mehta, Ph.D. P.Biol.
Study Director

98-12-04
Date

QUALITY ASSURANCE STATEMENT

Procedures, documentation, equipment records, etc., of the study (No. 970805/4) are examined in order to assure that the study is performed in accordance with the regulations specified as above and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures. This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Frank Kozar, Ph.D. P.Biol.
Director Quality Assurance

98-12-04
Date

TABLE OF CONTENTS

Page

Summary..... 5

Purpose..... 6

Characterization of Test and Control Articles..... 6

Materials and Methods..... 6

Results and Discussion..... 11

Conclusion..... 12

References..... 13

Data Tables 14

Appendix 1: Study Protocol..... 20

SUMMARY

The test article, Reactive Skin Decontamination Lotion, was tested for its ability to induce forward mutations conferring thioguanine resistant (TG^r) phenotype, at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus of the Chinese hamster ovary (CHO) cells. The cells of CHO-K1-BH_4 cell line were exposed, both in the presence and absence of the metabolic activation system (S9), to six dose levels (156, 313, 625, 1250, 2500 and 5000μg/ml) of the test article. The test article was solubilized in the culture medium, α Minimum Essential Medium (without the serum and the antibiotics) and dimethylsulfoxide (DMSO) was used as the solvent for positive control chemicals, ethyl methanesulfonate (EMS) and benzo(a)pyrene (BaP).

Cytotoxic effect of the test article was negligible at the dose range tested as relative clonal efficiency was greater than 70% in both, non-activated as well as S9-activated systems.

The frequency of TG^r mutants in the negative control group was within the range of published solvent control values (0 - $20/10^6$ clonable cells). The positive controls, EMS and BaP produced significant (in excess of 15 and 20-fold increase, respectively) positive response for TG^r mutants over the solvent control values. Thus, the positive and negative controls fulfilled the requirements for a valid test.

There was no apparent dose related response for the TG^r mutant frequency at the dose range tested and none of the doses showed greater than 20 TG^r mutant /10^6 clonable cells, both in the presence and the absence of the metabolic activation system.

Under the conditions of the assay described in this report and based on the evaluation criteria applied, Reactive Skin Decontamination Lotion elicited a negative (non-genotoxic) response in the *in vitro* mammalian CHO/HGPRT forward gene mutation assay.

PURPOSE

The purpose of this study is to evaluate *in vitro* genotoxic potential of the test article, Reactive Skin Decontamination Lotion (RSDL), based on the frequency of the induction of forward mutations at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in Chinese Hamster Ovary cells.

CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The test article, Reactive Skin Decontamination Lotion (RSDL), was received by Prairie Biological Research Ltd. on August 05, 1997 and assigned the code number 97-BZ-1.

Upon receipt, the test substance was described as bright yellow colored free flowing liquid with no distinct odor and was stored at 3 to 6°C in a tightly sealed opaque plastic container protected from moisture.

The culture medium, α Minimum Essential Medium (Life Technologies Cat. #12571-063) was used as a vehicle to dissolve and deliver the test article. The positive control compounds used in this assay are: benzo[a]pyrene (BaP; CAS 50-32-8) in the presence of metabolic activation (+S9) and ethyl methanesulfonate (EMS; CAS 62-50-0) in the absence of metabolic activation (-S9), supplied by Sigma Chemical Co. Dimethyl sulfoxide (DMSO, CAS 67-68-5) was used to solubilize and deliver BaP and EMS.

MATERIALS AND METHODS

Test System

The *in vitro* Chinese hamster ovary cell/hypoxanthine-guanine phosphoribosyl transferase (CHO/HGPRT) mutation assay has been established as a selective and sensitive test for the detection of a variety of chemical mutagens (Li et.al., 1987). The assay uses CHO-K_1-BH_4 cell line developed and extensively characterized by Hsie et.al. (1979). The cell line was obtained from Dr. A. W. Hsie, Department of Preventive Medicine and Community Health, University of Texas, Galveston, Texas, USA through Chrysalis, Scott Technology Park, Olyphant, PA, USA. The assay detects forward mutations in the X-linked hypoxanthine-guanine phosphoribosyl transferase (hgprt) locus (coding for the enzyme, HGPRT) in Chinese hamster ovary (CHO) cells. Presumptive mutants (thioguanine resistant, TG^r) due to the loss of HGPRT activity, are unable to convert the non-toxic purine analogue, 6-thioguanine (6TG) to its toxic metabolite (ribophosphorylated derivative) and, therefore, are resistant to its toxic effect. Wild-type cells, on the other hand, are sensitive to the toxic metabolite and are unable to grow in the presence of 6TG.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was obtained from Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, MD 20850, USA. The source of S9 is adult male Sprague-Dawley rats induced by a single intraperitoneal injection of Aroclor 1254 (@ 500 mg/kg) five days prior to sacrifice. The S9 is batch prepared and stored frozen approximately at -70° C until used. Each bulk preparation of S9 was assayed by the supplier for sterility and its ability to metabolize 2-aminoanthracene and 7,12-dimethyl-benz[a]anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S9 was thawed and mixed with cofactors to be added in sequence: 0.4ml of 0.5M magnesium chloride ($MgCl_2$), 0.4ml of 1.5M potassium chloride (KCl), 0.1ml of 1.0 M glucose-6-phosphate, 0.8 ml of 1 M nicotinamide adenine dinucleotide phosphate (NADP), 5.00 ml of 0.2 M disodium hydrogen phosphate ($Na_2 HPO_4$), 10.90 ml of distilled water, 2 ml of S9 homogenate, and 0.4 ml of calcium chloride ($CaCl_2$).

Solubility Test

In order of preference for the possible solvents compatible with the test system, the test article was first tested for solubility in the treatment medium. The test article was found to be readily soluble in the treatment medium, α minimum essential medium (αMEM) at a workable concentration of 500 mg/ml.

Preliminary Cytotoxicity Assay to Select Dose Levels

Cytotoxicity was measured as the reduction in the relative initial survival (cloning efficiency) of the cells following a 5-hour treatment with the test article in the presence (+S9) and the absence (-S9) of metabolic activation. Based on the cytotoxicity evaluation of the test article in an earlier study (study No. 970805/2 submitted to Defence Research Establishment) with the CHO-K_1 cell line, it was determined that the same dose range (313 to 5000 µg/ml) be used for the present study with CHO-K_1-BH_4 cell line. However, a cytotoxicity assay was run in parallel with but independent of the mutation assay and its results were reported as preliminary cytotoxicity assay. The highest dose (5000 µg/ml) represents the maximum dose recommended for the assay by OECD Chemical Testing Guideline (476).

CHO-K_1-BH_4 cell cultures initiated approximately 24 hours earlier were exposed to solvent, the complete medium (αMEM medium supplemented with 10% dialyzed fetal bovine serum, 100 units penicillin and 100 µg/ml streptomycin) alone and six dose levels (156, 313, 625, 1250, 2500, and 5000µg/ml) of the test article under non-activated and S9-activated conditions. The details of the treatment procedure are as described below

under the heading "Treatment of the Target Cells". Each treatment flask received 50µl dosing solution of test article. An untreated control was run in parallel. The cultures were exposed to the test article for 5 hours by incubating the flasks under standard conditions, at 37 ± 1° C in a humidified atmosphere of 5 ± 1% CO_2 in air. Following the treatment, cultures were washed twice with Saline G and refed with the complete medium (5 ml) and then incubated for about 20 hours under standard conditions. Saline G was prepared by mixing 2.2 g dextrose, 16.0 g NaCl, 0.8 g KCl, 0.58 g $Na_2HPO_4 .7H_2O$, 0.3 g KH_2PO_4, 0.308 g $MgSO_4 . 7H_2O$, 0.3 g $CaCl_2 .2H_20$ in one litre of distilled water and further diluting it by adding 1 litre of distilled water. The solution was sterilized through a prewashed (500 ml distilled water) 0.2 µm filter (Kao and Puck, 1969).

After 20-hr incubation, cultures were washed twice with Ca-Mg-free Saline G, harvested by trypsinization, and cells were counted by using trypan blue exclusion technique (Sigma-Aldrich, 1998). An aliquot of 200 cells of each culture was plated in duplicate in 60-mm culture dishes, each containing 5 ml of the complete medium. The dishes were incubated under standard conditions for about 7 days. On day 8, cells were fixed, stained, and colonies were counted.

Mutagenesis Assay

CHO-K_I-BH_4 cells were exposed to the vehicle alone, and six concentration of the test article in duplicate in both the absence and presence of metabolic activation system (S9). The preparation and addition of the test substance dosing solutions were carried out under golden light and the cultures were incubated in the dark during the exposure period.

Treatment of the Target Cells

CHO-K_I-BH_4 cells were cultured in 25-cm^2 flasks, each containing 5 ml of complete medium (αMEM medium containing 10% dialyzed fetal bovine serum, 2mM L-glutamine, 100 units penicillin and 100 µg/ml streptomycin) and an inoculum of approximately 8 x 10^5 cells. A set of duplicate cultures was used for each dose and all controls. Each flask is labeled with a preassigned study code that identifies the study number and treatment received. The flasks were incubated under standard conditions, at 37 ± 1° C in a humidified atmosphere of 5 ± 1% CO_2 in air, for about 24 hours. Logarithmically growing cultures were visually examined for growth and any contamination. At the end of incubation, medium was aspirated and cultures were washed twice with 5 ml of Saline G by gently tilting flask back and forth.

After the final wash, each flask received either 5 ml of pre-warmed serum free αMEM medium for non-activated (-S9) treatment conditions or 4 ml of the serum free complete medium plus 1 ml of S9 reaction mixture for treatment with activation (+S9). Cultures were exposed to freshly prepared test article doses (156, 313, 625, 1250, 2500, and

5000µg/ml), and positive and negative (vehicle) control chemicals. Each treatment flask received 50µl dosing solution of test article, or 5µl of each of the positive control chemicals (BaP or EMS] or the vehicle (DMSO). An untreated control was also run in parallel. The cultures were exposed to the test article for 5 hours by incubating the flasks under standard condition. After the treatment, cultures were washed twice with Saline G and refed with the complete medium (5 ml) and then incubated for about 20 hours under standard conditions.

Following 20-hr incubation, the medium was aspirated from the flasks and the cultures were washed twice with 5 ml Ca-Mg-free Saline G. The cultures were harvested in 2 ml trypsin (0.25% in HBSS, Sigma Cat.No. T-4424) followed by incubation at 37° C for 2-4 minutes to achieve complete detachment of cells. Then trypsin was inactivated by adding 2 ml complete medium to each flask. Cells were manually counted under the microscope by using the trypan blue exclusion technique. Cell suspensions were prepared in the medium and an appropriate number of cells were plated for evaluating cloning efficiency and subculturing for phenotypic expression of mutation.

Plating Cells for Relative Initial Survival (Cloning Efficiency)

For relative initial survival study, an aliquot containing 200 cells from each of the above cultures was plated in triplicate in three 60-mm culture dishes, each containing 5 ml of the complete medium. The dishes were incubated at standard conditions for 7 days. On day 8, cultures were fixed in 5 ml ethanol for 1min., air dried after discarding ethanol, and then stained with 5-10 ml 1% (v/v in water) crystal violet stain for 5 min or until desired staining of colonies obtained. The plates were washed in running tap water, air dried, and colonies were scored.

Subculturing for Phenotypic Expression of Mutation

For the phenotypic expression of mutation, the cells were passed through three subcultures, the first subculture occurring at the same time as plating for initial survival. An aliquot containing 1×10^6 cells was transferred to 100-mm culture dishes, each containing 10 ml complete medium and incubated at the standard conditions for 48 hours. All cultures were examined microscopically (inverted microscope) for monolayer establishment. Cultures were washed twice with 5 ml Ca-Mg-free Saline G after aspirating the medium, and harvested in 0.5 ml trypsin, which was subsequently inactivated with 1.5 ml complete medium. Cells were counted as described above and an aliquot of 1×10^6 cells from each culture was seeded to a 100-mm dish containing 10 ml complete medium to obtain a second subculture. The procedure was repeated to obtain a third subculture. The dishes were labeled with the study code, treatment conditions and other critical experimental parameters.

Selection of TG^r Mutants

The 100-mm dishes of the final subcultures were washed twice in Ca-Mg-free Saline G, and harvested in 0.5 ml trypsin, which was inactivated with 1.5 ml nucleoside-free αMEM medium (supplemented with the serum and the antibiotics). Cells were counted and cell suspension (1×10^6 cells/ml) of each culture was prepared in the same medium. Approximately 2×10^5 cells of each culture were plated in 5 (100-mm) replicate dishes, each containing 10 ml of the same nucleoside-free αMEM medium supplemented with 12.5 μM 6-thioguanine; and plates were incubated for about 7 days under standard conditions (O'Neill et. al., 1977). Then cultures were washed, and colonies were fixed in ethanol, stained and scored as previously described.

Cloning Efficiency

For determining cloning efficiency, 200 cells from the remaining of the above cell suspension were plated in triplicate in 60-mm dishes, each containing 5 ml nucleoside-free αMEM (supplemented with the serum and the antibiotics). The plates were incubated with the 100-mm dishes under standard conditions for about 7 days. Following incubation, cultures were washed, and colonies were fixed in ethanol, stained and scored.

Scoring Procedure

Plates were scored manually, using a Quebec Dark Field Colony Counter. Relative survival and mutant frequencies were determined.

Evaluation of Test Results

Evaluation of cytotoxic effects of the treatment is based upon relative initial survival (cloning efficiency). The number of colonies scored for each dose level was compared with the solvent control. The mutant frequency (expressed as TG^r mutants/10^6 clonable cells) was calculated as the total number of mutant colonies divided by the total number of cells plated and corrected for cloning efficiency at the time of mutant selection. The spontaneous mutant frequency is generally 0-10 TG^r mutasnt/10^6clonable cells, however values up to 20 are acceptable (Li et. al., 1987).

Test article was considered positive for mutagenicity, if it exhibited a dose dependent increase in mutant frequencies (as examined with Student's t-test and an analysis of variance, ANOVA) with at least two consecutive doses showing mutant frequencies, which are elevated above 40 mutants per 10^6 clonable cells. On the other hand, if none of the exposed cultures showed a mutant frequency above 40 mutants per 10^6 clonable cells, the test article was considered negative for mutagenic activity. The mutagenic response was considered suspect if there was an increase in mutant frequency above 40 mutants per 10^6 clonable cells at a single dose level.

Criteria for a Valid test

The following criteria were applied for judging the acceptability of the data of the assay:

1. The absolute cloning efficiency (number of colonies formed/number of cells plated) of the negative controls should not be less than 50%.

2. The mean mutant frequency of the solvent controls should fall within the range of 0 – 20 mutants per 10^6 clonable cells.

3. The positive control must induce mutant frequency that is at least three times that of the solvent control value and must exceed 40 mutants per 10^6 clonable cells.

Archives

Upon completion of the final report, all raw data and reports are maintained in Prairie Biological Research Ltd.'s Archives located at 4290-91A Street, Block C, Edmonton, Alberta, T6E 5V2, Canada.

RESULTS AND DISCUSSION

Solubility test

The treatment medium (αMinimum Essential Medium) was determined to be the solvent of choice based on the solubility of the test article. The test article was readily soluble in the treatment medium at the concentration of 500 mg/ml, the maximum working stock concentration used in the assay.

Preliminary Cytotoxicity Assay

The results from the cytotoxicity assay, which was conducted in parallel with the mutation assay, are presented in Table 1 (for non-activated system) and Table 2 (for S9-activated system). The cells were exposed to six dose levels (156, 313, 625, 1250, 2500 and 5000 µg/ml) under non-activated (-S9) and activativated (+S9) conditions. The osmolality of the solvent, culture medium (αMEM) was 266 mOsm/kg and the osmolality of the highest dose, 5000 µg/ml, was 328 mOsm/kg. The pH of the test article stock solution (pH 10.0) was adjusted to 7.0 with 1M HCl.

Cytotoxicity, as measured by clonal efficiency (CE), was approximately 20 % (i.e. 80% CE) in the non-activated system and 26% (74% CE) in the S9-activated system at the

highest dose level tested. Thus, the dose range tested was considered as optimal for the mutation assay.

TG^r Mutant Selection

The assay was conducted in both the absence and presence of an Aroclor-induced S9 activation system at dose levels of 156, 313, 625, 1250, 2500 and 5000 µg/ml. Cells were exposed to the test article for 5 hours followed by a 20-hour recovery period under standard conditions.

For the non-activated system, the data on individual cultures are presented in Table 3 and the results are summarized in Table 5. The absolute cloning efficiency was in excess of 94% for the solvent control. The maximum cytotoxicity in the mutation assay was observed at the highest dose level (5 mg/ml), the cloning efficiency being about 66%. Although, the frequency of TG^r mutants in the solvent control was $11.6/10^6$ (i.e. $>10/10^6$) clonable cells, it was considered as insignificant to affect the assay (Li, *et al.*, 87). Since the mean mutant frequency of the test article for all doses was within the recommended range 0 – 20 mutants/10^6 clonable cells, no statistical test was performed on the data. A dose related response for mutation induction was not apparent from the data. The frequency of TG^r mutants for EMS (positive control) was $176.2/10^6$ clonable cells, which is significantly (in excess of 15-fold) higher than the solvent control.

For S9-activated system, the data on individual cultures are presented in Table 4 and the results are summarized in Table 6. The absolute cloning efficiency was in excess of 98% for the solvent control. The maximum cytotoxicity was recorded at the highest dose level, which showed a cloning efficiency value of about 73%. Although the frequencies of TG^r colonies were in excess of the solvent control ($3/10^6$ clonable cells) at the six dose levels tested, the dose related response was not apparent. More over, the mutant frequencies were within the range (0 – 20) of published solvent control data (Li, *et.al.*, 87), therefore no statistical test was performed. The TG^r mutant frequency for the positive control (Benzo[a]pyrene) was $65.7/10^6$ clonable cells, a significant (in excess of 20-fold) increase over the solvent control value.

CONCLUSION

The positive and the negative controls fulfilled the requirements for a valid test. Under the conditions of the assay described in this report and based on the evaluation criteria applied, the test article, Reactive Skin Decontamination Lotion was found to exhibit a negative (non-genotoxic) response in the presence as well as absence of the S9 metabolic activation in the CHO/HGPRT forward mutation assay.

REFERENCES

1. **Hsie, A.W. , J.P. O'Neill, D.B. Couch, J.R. SanSebastian, P.A. Brimer, R. Machanoff, J.C. Riddle, A.P. Li, J.C. Fuscoe, N.L. Forbes and M.H. Hsie (1979).** Utilization of a quantitative mammalian cell mutation system in experimental mutagenesis and genetic toxicology, in: B. Butterworth (Ed.), Strategy for Short-Term Testing for Mutagens/Carcinogens, C.R.C. Press, West Palm Beach, pp. 39-54.

2. **Li, A.P., J.H. Carver, W.N. Choy, A.W. Hsie, R.S. Gupta, K.S. Loveday, J.P. O'Neill, J.C. Riddle, L.F. Stankowski Jr. and L.L. Yang (1987).** A guide to the performance of the Chinese Hamster Ovary cell/hypoxanthine-guanine phosphoribosyl transferase gene mutation assay, *Mutation Research*, 189: 135 - 141.

3. **Sigma-Aldrich Canada Ltd., Catalogue 1998.** Use of Trypan Blue stain and the hemocytometer to determine total cell count and viable cell number, p. 1844.

4. **O'Neill, J.P., P.A. Brimer, R. Machanoff, G.P. Hirch and A.W. Hsie (1977).** A quantitative assay of mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese Hamster Ovary cells (CHO/HGPRT system); development and definition of the system, *Mutation Research*, 59: 109-118.

Table 1 CHO/HGPRT Mutation Assay

Preliminary Cytotoxiclty Test (Without S9)

Treatment ug/ml	Colonies Per Dish[1]			Cloning Efficiency[2]	Relative Cloning Efficiency %[3]
	1	2	3		
Solvent Control[4]	198	207	211	1.03	100
RSDL Article[5]					
156 ug/ml	179	199	220	1.00	96.8
313 ug/ml	186	188	192	0.94	91.6
625 ug/ml	198	197	177	0.95	92.5
1250 ug/ml	163	163	172	0.83	80.7
2500 ug/ml	178	187	185	0.92	89.0
5000 ug/ml	171	168	156	0.83	80.1
Positive Control:					
DMSO[6]	194	194	203	0.99	95.6
EMS[7]: 200 ug/ml	224	149	187	0.93	90.6

1. Colonies per Dish = 200 cells plated per dish
2. Absolute Cloning Efficiency = Total colonies in 3 dishes /total number of cells inoculated
3. Relative Cloning Efficiency % = (Cloning Efficiency of treatment/cloning efficiency of solvent control) x 100
4. Solvent Control = α Minimum Essential Medium (α MEM)
5. RSDL = Reactive Skin Decontamination Lotion, the test article
6. DMSO = Dimethyl sulfoxide: solvent for positive control
7. EMS = Ethyl methanesulfonate

Table 2

CHO/HGPRT Mutation Assay

Preliminary Cytotoxicity Test (With S9)

Treatment µg/ml	Colonies per dish[1]			Cloning Efficiency[2]	Relative Cloning Efficiency %[3]
	1	2	3		
Solvent Control[4]	223	241	211	1.13	100.0
RSDL Article[5]:					
156 µg/ml	198	175	210	0.97	85.8
313 µg/ml	192	186	188	0.94	83.2
625 µg/ml	186	186	202	0.96	85.0
1250 µg/ml	169	185	175	0.88	77.9
2500 µg/ml	182	170	198	0.92	81.4
5000 µg/ml	154	183	166	0.84	74.3
Positive Control:					
DMSO[6]	189	155	168	0.85	100.0
Benzo[a] pyrene:					
4µg/ml	174	149	129	0.75	88.2

1. Colonies per Dish = 200 cells plated per dish
2. Absolute Cloning Efficiency Tctal colonies in 3 dishes /total number of cells inoculated
3. Relative Cloning Efficiency % = (Cloning Efficiency of treatment/cloning efficiency of solvent control) x100
4. Solvent Control = α Minimum Essential Medium (αMEM)
5. RSDL = Reactive Skin Decontamination Lotion, the test article
6. DMSO = Dimethyl sulfoxide - solvent for positive control

Table 3 CHO/HGPRT mutation Assay
Concurrent Cytotoxicity Assay (Without S9)

Treatment µg/ml	Colonies per dish[1]			Cloning Efficiency[2]	Relative Cloning Efficiency[3] %
	1	2	3		
Solvent Control[4]	198	196	188	0.97	100.0
RSDL Article[5]					
156 µg/ml	198	178	197	0.96	98.5
313 µg/ml	190	177	159	0.88	90.4
625 µg/ml	166	181	168	0.86	88.5
1250 µg/ml	154	144	161	0.77	78.9
2500 µg/ml	145	143	136	0.71	72.9
5000 µg/ml	122	129	132	0.64	65.8
Positive Control:					
DMSO[6] control	211	195	189	0.99	100.0
EMS[7]: 200µg/ml	183	179	176	0.90	90.0

1. Colonies per Dish = 200 cells plated per dish
2. Absolute Cloning Efficiency = (Total cell colonies in 3 dishes /total number of cells inoculated)
3. Relative Cloning Efficiency % = (Cloning Efficiency of treatment/solvent control cloning efficiency) x 100
4. Solvent Control = α Minimum Essential Medium (α MEM)
5. RSDL = Reactive Skin Decontaminant Lotion, the test article
6. DMSO = Dimethyl sulfoxide -solvent for positive control
7. EMS = Ethyl methanesulfonate

Table 4 CHO/HGPRT mutation Assay
Concurrent Cytotoxicity Assay (With S9)

Treatment µg/ml	Colonies per dish[1]			Cloning Efficiency[2]	Relative Cloning Efficiency %[3]
	1	2	3		
Solvent Control[4]	188	185	174	0.91	100.0
RSDL Article[5]					
156 µg/ml	173	177	172	0.87	95.4
313 µg/ml	184	168	149	0.84	91.2
625 µg/ml	169	168	154	0.82	89.8
1250 µg/ml	177	143	144	0.77	84.2
2500 µg/ml	136	137	136	0.68	74.8
5000 µg/ml	124	122	151	0.66	72.6
Positive Control:					
DMSO control[6]	195	183	175	0.92	100.0
Benzo[a] pyrene:					
4 µg/ml	126	136	121	0.64	69.3

1. Colonies per Dish = 200 cells plated per dish
2. Absolute Cloning Efficiency = (Total cell colonies in 3 dishes /total number of cells inoculated)
3. Relative Cloning Efficiency % = (Cloning Efficiency/solvent control cloning efficiency) x 100
4. Solvent Control = α Minimum Essential Medium (αMEM)
5. RSDL = Reactive Skin Decontamination Lotion, the test article
6. DMSO = Dimethyl sulfoxide: solvent for positive control

Table 5

CHO/HGPRT mutation Assay of RSDL (without S9)

Treatment µg/ml	Colonies per dish[1]			Total Colonies	Cloning Efficiency[2]	Mutant Colonies/Selection Dish					Total Mutant Colonies	TG^r Mutant/10^6 Clonable Cells[3]
	1	2	3			1	2	3	4	5		
Solvent Control[4]	194	195	178	567	0.945	1	2	2	0	0	5	11.6
RSDL Article[5]:												
156 µg/ml	174	177	186	537	0.895	1	2	1	0	0	4	4.5
313 µg/ml	177	184	169	530	0.883	2	2	0	2	0	6	5.35
625 µg/ml	166	183	188	537	0.895	0	1	0	0	0	1	1.1
1250 µg/ml	169	163	176	508	0.847	0	0	0	0	0	0	0
2500 µg/ml	184	158	159	501	0.835	3	3	2	0	3	11	12.4
5000 µg/ml	115	146	118	379	0.632	3	3	4	0	1	11	16.4
Positive Control:												
DMSO[6]	146	142	173	461	0.768	0	0	0	0	0	0	0
EMS[7]: 200µg/ml	123	139	106	368	0.613	17	20	13	27	31	108	176.2

1. Colonies per dish=200 cells plated per dish
2. Absolute Cloning Efficiency = Counted colonies/initial number of cells plated
3. TG^r mutant frequency = (total number of mutant colonies scored)/ total number of cells plated x cloning efficiency), expressed as per 10^6 clonable cells
4. Solvent Control = α Minimum Essential Medium (α MEM)
5. RSDL = Reactive Skin Decontamination Lotion, the test article
6. DMSO = Dimethyl sulfoxide, solvent for EMS
7. EMS = Ethyl methanesulfonate

Table 6

CHO/HGPRT mutation Assay of RSDL (with S9)

Treatment µg/ml	Colonies per dish[1]			Total Colonies	Cloning Efficiency[2]	Mutant Colonies/Selection Dish					Total Mutant Colonies	TG^r Mutant/10^6 Clonable Cells[3]
	1	2	3			1	2	3	4	5		
Solvent Control[4]	179	202	211	592	0.987	1	1	1	0	0	3	3
RSDL Article[5]												
156 µg/ml	189	198	193	580	0.967	1	3	1	3	0	8	8.3
313 µg/ml	200	216	168	584	0.973	2	0	1	0	1	4	4.1
625 µg/ml	166	191	195	552	0.92	1	1	0	3	1	6	6.5
1250 µg/ml	154	174	173	501	0.835	2	1	0	0	3	6	7.2
2500 µg/ml	200	157	182	539	0.898	1	1	1	0	1	4	4.5
5000 µg/ml	162	178	172	512	0.853	4	2	2	3	2	13	15.2
Positive Control:												
DMSO[6]	189	215	219	623	1.038	0	0	0	0	1	1	0.96
Benzo[a]pyrene: 4 µg/ml	164	157	184	505	0.842	9	9	10	14	14	17	65.7

1. Colonies per dish = 200 cells plated per dish
2. Cloning Efficiency = Counted colonies/Initial number of cells plated (200)
3. TG^r mutant frequency =(total number of mutant colonies scored)/(total number of cells plated x clonal efficiency), expressed as per 10^6 clonable cells.
4. Solvent Control = α Minimum Essential Medium (α MEM)
5. RSDL = Reactive Skin Decontamination Lotion, the test article
6. DMSO = Dimethyl sulfoxide, solvent for positive control

APPENDIX 1 : STUDY PROTOCOL

STUDY PLAN

CHINESE HAMSTER OVARY CELL/HYPOXANTHINE–GUANINE PHOSPHORIBOSYL TRANSFERASE (CHO/HGPRT) GENE MUTATION ASSAY

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of a test article identified as Reactive Skin Decontamination Lotion (RSDL) based upon its ability to induce mutations at hypoxanthine-guanine phosphoribosyl transferase locus (HGPRT) locus in Chinese hamster ovary (CHO) cells.

2.0 SPONSOR

2.1 Name: Defence Research Establishment

2.2 Address: P.O. Box 4000
Medicine Hat, Alberta
T1A 8K6

2.3 Representative Dr. Thomas Sawyer

2.4 Sponsor Project #: W7702-6-R599/A

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: Reactive Skin Decontamination Lotion (RSDL)
NSN4230-21

3.2 Controls:

Untreated:	Untreated cells
Negative:	Test article solvent
Positive:	Benzo-a-pyrene, and Ethyl methanesulphonate

3.3 Determination of Strength, Purity, etc.

The sponsor will be directly responsible for: (a) determination and documentation of the analytical purity and composition of the test article, and (b) the stability and strength of the dosing solutions.

P&R

3.4 Test article Sample Retention

The Sponsor will be responsible to retain a reserve sample of the test article.

4.0 TESTING LABORATORY & KEY PERSONNEL

4.1 Name: Prairie Biological Research Ltd.

4.2 Address: 4290-91A Street
Edmonton, Alberta
Canada, T6E 5V2

4.3 Study Director: Ram D. Mehta, Ph.D. P.Biol.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Starting Date: September 15, 1998

5.2 Proposed Experimental Completion Date: October 23, 1998

5.3 Proposed Report Date: October 25, 1998

6.0 TEST SYSTEM

The CHO-K_1-BH_4 cell line was supplied by Dr. A.W. Hsie, Department of Preventive Medicine and Community health, University of Texas, Galveston, USA through Chrysalis, Preclinical Services-North America. This system has been demonstrated to detect the mutagenic activity of a variety of chemicals (Hsie et.al., 1979; Li et. al., 1987). The assay detects forward mutations in the X-linked hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in Chinese hamster ovary (CHO) cells. The mutations at this locus produce thioguanine resistant (TG^r) phenotype due to the loss of the enzyme, HGPRT.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The gene mutation assay will be conducted using standard procedures by exposing cultures of CHO cells to a minimum of six concentrations of the test article (based on its cytotoxic effect and solubility limit) as well as positive and solvent controls according to the method described by Li et. al. (1987). The cells in the logarithmic phase of growth will be exposed to the test article doses in the presence and absence of a metabolic activation system (S9) for 5 hrs under standard conditions, at 37 $\pm$ 1° C in a humidified atmosphere of 5 $\pm$ 1% CO_2 in air. Following the treatment, cultures will be washed and refed with fresh growth medium and then incubated overnight under standard conditions for post–treatment recovery. Cells will be harvested by trypsinization and counted to prepare cell suspensions of appropriate densities to evaluate relative initial survival (cloning efficiency) and to obtain subcultures for phenotypic expression of mutation. For determining cloning efficiency, cells are plated in the growth medium and for mutation selection they are plated in the selective medium containing 6-thioguanine.

7.1 Selection of Solvent

Unless the Sponsor has specified the test article solvent, a solubility determination will be conducted to determine the maximum soluble concentration (up to a maximum of 500mg/ml) in a variety of solvents compatible with the test system. Solvents compatible with this test system, in order of preference, include but are not limited to, culture medium or distilled water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5),

ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The solvent of choice for the test article will be the one, selected in order of preference that permits preparation of the highest stock concentration, up to a maximum of 500 mg/ml.

7.2 Dose Selection

A prescreen cytotoxicity evaluation of the test article will be performed for the selection of doses to be evaluated in the mutation assay. Cytotoxicity of the test article in CHO cells will be determined by a reduction in the relative initial survival of the cells following a 5-hour treatment with the test article in the presence and absence of metabolic activation system (S9). The highest concentration of test article evaluated in the mutation assay will be either that which results in approximately 10 % relative cell survival (vs. the untreated or solvent controls) or the one set by its solubility limit or 5 mg/ml as recommended in the OECD Chemical Testing Guideline 476.

7.3 Activation System

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The source of S9 will be adult male Sprague-Dawley rats induced by a single intraperitoneal injection of Aroclor 1254 @ 500 mg/kg, five days prior to sacrifice. The S9 will be batch prepared and stored frozen approximately at -70° C until used. Alternatively, the S9 prepared under these specifications will be obtained from a reputable commercial supplier.

Immediately prior to use, the S9 will be thawed and mixed with cofactors to be added in sequence: 0.4 ml of 0.5M magnesium chloride ($MgCl_2$), 0.4 ml of 1.5M potassium chloride (KCl), 0.1 ml of 1.0 M glucose-6-phosphate, 0.8 ml of 1 M nicotinamide adenine dinucleotide phosphate (NADP), 5.00 ml of 0.2 M disodium hydrogen phosphate ($Na_2 HPO_4$), 10.90 ml of distilled water, 2 ml of S9 homogenate, and 0.4 ml of calcium chloride ($CaCl_2$). The mixture will be sterilized by membrane (0.2 µm) filtration and stored at 4° C prior to use.

7.4 Controls

7.4.1 Untreated Control

Cells not exposed to the test article will be used as the untreated control.

7.4.2 Solvent Control

The solvent for the test article will be used as the solvent control. For solvents other than water, physiological buffer or culture medium, the final concentration in the treatment medium will not exceed 1%.

7.4.3 Positive Control

Ethyl methanesulphonate will be used at a concentration of 200 µg/ml as the positive control in the non-activated study. Benzo[a]pyrene will be used at a concentration of 1-4 µg/ml as the positive control in the S9-activated study.

7.5 Plating cells for Treatment

Exponentially growing CHO-K_1-BH_4 cells will be seeded in complete medium, αMEM (minimum essential medium) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin/ml and 100 µg/ml streptomycin. For each treatment condition and controls, approximately 0.8 x 10^6 cells/25 cm^2 tissue culture flask containing 5 ml complete medium (αMEM). Duplicate flasks will be prepared for each treatment dose and control. The flasks will be incubated under standard condition, at 37± 1° C in a humidified atmosphere of 5± 1% CO_2 in air for 16-24 hours.

7.6 Labeling Procedure for Identification

All culture flasks and dishes will be marked with a permanent marking pen by a pre-assigned PBR study number and a code system to identify the treatment condition, replicate number and other critical test parameters.

7.7 Treatment of Cells with Test or Control Articles

Logarithmic growing cultures (section 7.5) will be visually inspected for normal growth and any contamination. At the end of incubation period, medium will be aspirated, and the cultures will be washed twice with 4-5 ml of Saline G by gently tilting flask back and forth. Saline G is prepared by mixing 2.2 g dextrose, 16 g NaCl, 0.8 g KCl, 0.58 g $Na_2HPO_4 .7H_2O$, 0.3 g KH_2PO_4, 0.308 g $MgSO_4 . 7H_2O$, 0.3 g $CaCl_2 . 2H_20$ in one litre of distilled water and further diluting it by adding 1 litre of distilled water. The solution will be sterilized through a prewashed (500 ml distilled water) 0.2 µm filter (Kao and Puck, 1969).

After washing, each culture will be refed with 5 ml of the complete medium for the non-activated exposure or 4 ml S9 mixture plus 1 ml complete medium for the S9-activated exposure. Each treatment flask will receive equal volume of test article or solvent alone. An untreated control (cells either in the complete medium alone or in the medium plus S9 mixture) will also be included. The positive control chemicals specific for the presence (+S9) and absence (-S9) of metabolic activation will be included.

The cells will be treated for 5 hours under standard condition. At the end of the treatment period, the medium will be aspirated, the cultures washed with saline G, refed with 5 ml complete medium and incubated overnight under standard conditions. These cultures will be prepared, as described below, for plating to estimate relative initial survival (or cytotoxicity) and subculturing for phenotypic expression of mutation.

7.8 Plating Cells for Relative Initial Survival

Following overnight incubation after treatment, the medium will be aspirated and cultures will be washed twice with Ca-Mg-free Saline G. The individual cultures will be harvested in 2 ml trypsin (0.25% in HBSS, Sigma Cat. No.T-4424) followed by incubation at 37° C for 2-4 minutes to achieve complete detachment of cells. Trypsin is inactivated by adding 2 ml complete medium to each flask. Cell densities will be determined for each culture by trypan blue exclusion technique (Sigma-Aldrich, 1998). An aliquot of the cells will be diluted to a cellular density of 1000 cells/ml in Ca-Mg-free Saline G , and 0.2 ml (200 cells) of each suspension will be plated in triplicate in 60-mm plates, each containing 5 ml complete medium. The plates will be incubated for 7-10 days under standard conditions and cultures will be washed, fixed, stained and scored.

7.9 Subculturing Cells for Phenotypic Expression of Mutation

For the phenotypic expression of mutation, the treated cells will undergo three subcultures, the first subculture occurring at the same time as plating for initial survival. For each subculture, an aliquot of cell suspension containing $1x\ 10^6$ cells will be transferred to a 100-mm dish containing 10 ml of complete medium. The plates will be incubated for 2-3 days under standard conditions. Following incubation, cultures will be washed twice with Ca.-Mg-free Saline G and harvested in 0.5 ml trypsin that will be inactivated by adding 1.5 ml complete medium after allowing 2-3 minutes incubation at 37° C to achieve complete detachment of cells. Cell density of each suspension will be determined and an aliquot of $1x\ 10^6$ cells will used for the next subculture. Additional subcultures will be performed on Days 3 and 5 with selection on Day 8.

7.10 Selection of Mutant (TG^r) Cells

The 100-mm dishes of the final subcultures will be washed twice in Saline G and harvested in 0.5 ml trypsin, which will be subsequently inactivated with 1.5 ml nucleoside free αMEM (supplemented with the serum and the antibiotics). Cells will be counted and cell suspension (1×10^6 cells/ml) of each culture will be prepared in

the same medium. An aliquot of 2 ml of 1:10 dilution of each cell suspension (approximately 2 x 10^5 cells) will be plated in each of 5 (100-mm) replicate dishes, each containing 8 ml complete αMEM (nucleoside free medium supplemented with 12.5 μM 6-thioguanine) followed by incubation for about 7-10 days under standard conditions (O'Neill et. al., 1977). Cultures will be washed, and colonies will be fixed, stained and scored for TG^r mutant colonies as described below (section 7.11).

7.10.1 Cloning Efficiency

From the remaining cell suspension (1 x 10^5 cells/ml) of the final subcultures, 0.10 ml will be diluted to a cellular density of 1 x 10^3 cells/ml. An aliquot of 0.2 ml (200 cells) of this suspension will be plated in each of three 60-mm dishes containing 5 ml of complete medium. These plates will be incubated under standard conditions for 7-10 days with the 100-mm selection plates. Colonies will be fixed, stained and scored for determining cloning efficiency as described below (section 7.11).

7.11 Fixing and Staining and Scoring

Following incubation for 7-10 days, the medium will be removed from the plates and discarded. The colonies will be fixed by adding 5-10 ml of ethyl alcohol to each plate for 1 min. After discarding ethyl alcohol, plates will be stained by the addition of 5-10 ml dilute crystal violet solution (~1% v/v in water) for 5 minutes or until desired staining of colonies is obtained as judged by visual inspection. The stain will be removed from the plates and the plates will be rinsed under running water to remove any residual stain and air dried. Colonies will be scored using either a BioTran II Automated Colony Counter (Model C111) or manually with a Quebec Darkfield Colony Counter.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria will be applied for judging the acceptability of the data of the assay:

1. The absolute cloning efficiency (number of colonies formed/number of cells plated) of the negative controls should not be less than 50%.

2. The mean mutant frequency of the solvent controls should fall within the range of 0 – 20 mutants per 10^6 clonable cells.

3. The positive control must induce mutant frequency that is at least three times that of the solvent control value and must exceed 40 mutants per 10^6 clonable cells.

9.0 EVALUATION OF TEST RESULTS

Evaluation of cytotoxic effects of the treatment will be based upon relative initial survival (cloning efficiency). The number of colonies scored for each dose level will be compared with the solvent control. The mutant frequency (expressed as TG^r mutants/10^6 clonable cells) will be calculated as the total number of mutant colonies divided by the total number of cells plated and corrected for cloning efficiency at the time of mutant selection. The spontaneous mutant frequency is generally 0-10 TG^r mutant/10^6clonable cells, however values up to 20 are acceptable (Li et. al., 1987). The data will be presented in the final study report in tabular form for absolute and relative cloning efficiency and TG^r mutant frequency/10^6clonable cells.

A test article will be considered positive for mutagenicity if it exhibited a dose dependent increase in mutant frequencies (as examined with Student's t-test and an analysis of variance, ANOVA) with at least two consecutive doses showing mutant frequencies, which are elevated above 40 mutants per 10^6 clonable cells. On the other hand, if none of the exposed cultures showed a mutant frequency above 40 mutants per 10^6 clonable cells, the test article will be considered negative for mutagenic activity. The mutagenic response will be judged as suspect if there is an increase in mutant frequency above 40 mutants per 10^6 clonable cells at a single dose level.

10.0 REPORT

A report of the results of this study will be prepared by Prairie Biological Research Ltd. and will accurately describe all methods used for generation and analysis of data.

Results presented will include, but not be limited to:

- description of the cells used;
- test conditions: composition of media, CO_2 environment, exposure period, concentration of test article and rationale for selection of dose levels, type of metabolic activation system used, positive, untreated, and solvent controls;
- absolute and relative cloning efficiency (method for determination; data given separately for each culture);
- historical or published control data;
- criteria used for establishing the validity of the test data;
- criteria for evaluating test results;
- discussions of the results;
- conclusion;
- summary, and;
- quality control report, if requested.

11.0 RECORDS AND ARCHIVES

11.1 Records

Upon completion of the final report, all raw data and reports will be maintained by the Quality Assurance Unit of Prairie Biological Research Ltd. 4290-91A Street, Edmonton, Alberta, Canada in accordance with the relevant Good Laboratory Practice Regulations.

11.2 Specimens

All specimens will be held in storage in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written in accordance with OECD Guideline for the Testing of Chemicals, 476, July 1997.

This study will be performed in compliance with the provisions of the OECD Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? YES

If so, to which agency or agencies? HEALTH CANADA

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and the residual test article will be disposed of following finalization of the report.

13.0 APPROVAL

T.W. Sawyer — 17 Dec 98

Sponsor Representative (Signature) — Date

T.W. SAWYER

Name (Print or Type)

[signature] — Sept 1, 1998

PBR Study Director (Signature) — Date

Dr. Ram D. Mehra

Name (Print or Type)

14.0 REFERENCES

1. Hsie, A.W. , J.P. O'Neill, D.B. Couch, J.R. SanSebastian, P.A. Brimer, R. Machanoff, J.C. Riddle, A.P. Li, J.C. Fuscoe, N.L. Forbes and M.H. Hsie (1979). Utilization of a quantitative mammalian cell mutation system in experimental mutagenesis and genetic toxicology, in: B. Butterworth (Ed.), *Strategy for Short-Term Testing for Mutagens/Carcinogens*, C.R.C. Press, West Palm Beach, pp. 39-54.

2. Kao, F.T. and T.T. Puck 1967) Genetics of somatic mammalian cells. IV. Properties of Chinese hamster cell mutants with respect to the requirement for proline, *Genetics*, 55: 513-518.

3. Li, A.P., J.H. Carver, W.N. Choy, A.W. Hsie, R.S. Gupta, K.S. Loveday, J.P. O'Neill, J.C. Riddle, L.F. Stankowski Jr. and L.L. Yang (1987). A guide to the performance of the Chinese Hamster Ovary cell/hypoxanthine-guanine phosphoribosyl transferase gene mutation assay, *Mutation Research*, 189: 135- 141.

4. O'Neill, J.P., P.A. Brimer, R. Machanoff, G.P. Hirch and A.W. Hsie (1977). A quantitative assay of mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese Hamster Ovary cells (CHO/HGPRT system); development and definition of the system, *Mutation Research*, 59: 109-118.

5. Sigma-Aldrich Canada Ltd., Catalogue (1998). Use of Trypan Blue stain and the hemocytometer to determine total cell count and viable cell number, p. 1844.